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Cadmium directly acts on endothelin receptor and inhibits endothelin binding activity

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The binding of endothelin (ET) to human placenta ET receptor was strongly inhibited by cadmium ions (Cd^{2+}) ($IC_{50} = 2 \times 10^{-5}$ M). Experiments with affinity cross-linking showed that the major 40 kDa receptor was inhibited to form a [^{125}I]ET-1/receptor complex. The mode of inhibition was noncompetitive with respect to ET-1. The inhibitory effect of Cd^{2+} on solubilized ET receptor was partially reversed by the chelating agent, ethylenediaminetetraacetic acid (EDTA), whereas the effect was irreversible for the membrane-associated receptor. The rat aorta contractions by ET were prevented by pretreatment or addition of Cd^{2+}.

Vaso constriction; Hypertension; Hypotension; Human placenta; Divalent metals; Chelating agent

1. INTRODUCTION

Endothelin (ET) is a potent vasoconstrictive peptide produced by endothelial cells of blood vessels [1,2]. The pressor response of ET is largely ascribable to its vasoconstrictive activity [1–7]. This activity is greater than that of any other known vasoconstrictors of mammalian origin including human tissue and is apparently correlated with the high affinity of endothelin to its receptor. The action of ET has been shown to increase the intracellular calcium (Ca^{2+}) concentration [1,2]. Specific binding sites for ET have been detected in various animal cells and tissues [8–13]. The 40 kDa receptor which we have previously solubilized and purified from human placenta is one of them [14]. In this study, the effects of cadmium (Cd^{2+}) ion on the binding of ET to receptor and the rat aorta contraction induced by ET were investigated.

2. MATERIALS AND METHODS

2.1. *Materials*

Human ET-1, ET-2 and ET-3 were purchased from Peptide Institute Inc. (Osaka, Japan), and ^{125}I-labeled ET-1 (spec. act. 62 Tbq/mmol) was from Amersham. All other reagents were of analytical grade. Divalent metals used in the present experiments are all in chloride form.

2.2. *Preparation of endothelin receptor*

Human placenta membrane was prepared by the method described previously [14]. For preparation of solubilized receptor, it was homogenized with 50 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid)) buffer (pH 7.4) containing 0.6% zwitterionic detergent CHAPS (3-[(3-cholamidopropyl)]dimethylammonio-propane sulfonic acid), 0.6 M NaCl and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The ET receptor was solubilized by stirring the mixture overnight at 4°C. The mixture was centrifugated at 21 000 × *g* for 60 min and ET receptor was obtained in the supernatant fraction. The solubilized receptor (10 ml) was applied to Sephadex G-25 column (1.5 × 40 cm) equilibrated with 50 mM HEPES buffer (pH 7.4), 0.1% CHAPS and 0.5 mM PMSF, and the fractions containing ET binding activity were pooled.

2.3. *Binding assay*

The binding assay was carried out according to the previous report [14] with slight modifications. The reaction mixture (50 μl) contained 50 mM HEPES buffer (pH 7.4), 0.1% CHAPS, solubilized ET receptor (about 2 μg of protein) and 62.5 pM [^{125}I]ET (~1 × 10^4 cpm). It was incubated for 30 min at room temperature (25°C). Nonspecific binding was determined by the addition of 500 nM nonradioactive ET-1. The receptor [^{125}I]ET complex was separated from free [^{125}I]ET by mixing 50 μl of 15% (v/v) calcium phosphate gel which precipitated the complex upon centrifugation (15 000 rpm, for 1 min). The pellet was washed with 0.3 ml of 50 mM sodium phosphate buffer (pH 7.4)/0.2% CHAPS, and reprecipitated by centrifugation (15 000 rpm, 1 min). The radioactivity was counted with a gamma-counter.

2.4. *Cross-linking of [^{125}I]ET receptor*

An aliquot (10 μl) of the solubilized ET receptor (2 mg protein/ml) was incubated for 30 min with 400 pM [^{125}I]ET-1 in 50 μl of 50 mM HEPES buffer (pH 7.4) containing 0.1% CHAPS. To the reaction mixture was added 0.5 mM disuccinimidyl suberate (DSS) and was further incubated for 40 min at room temperature. After addition of Tris-HCl (pH 6.8) to final 60 mM, the cross-linked receptor was analyzed by 11% polyacrylamide gel electrophoresis in the presence of 1% sodium dodecylsulfate (SDS).

2.5. *Vasoconstriction assay*

Thoracic aortae were isolated from anesthetized male Wistar rats (~200 g). The aortae were cut into helical strips, suspended in Krebs-Ringer solution which were maintained at 37°C and gassed with 95% O_2/5% CO_2. Tensions, developed by the addition of ET-1, were recorded with force/displacement transducers.

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3. RESULTS

Among the various heavy metals tested, Cu^{2+} and Cd^{2+} strongly inhibited the binding of [^{125}I]ET-1 to the solubilized endothelin receptor from human placenta (Table I). The binding was not inhibited by Fe^{2+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+} or Ni^{2+} even at high concentrations (10 mM). To know more about the inhibition by Cd^{2+} and Cu^{2+}, a detailed study was undertaken with decreasing concentrations of the two divalent cations. The results showed that inhibitory effect of Cd^{2+} on ET-1 binding (50% inhibitory concentration, $IC_{50} = 20$ μM) was greater than that of Cu^{2+} (200 μM).

We have previously shown that human placenta contained a major 40 kDa receptor which was affinity labeled by cross-linking with [^{125}I]ET-1, -2, and -3 [14]. As shown in Fig. 1, the [^{125}I]ET-1 cross-linking to 40 kDa receptor was diminished to about 50% and 90% in the presence of 20 μM and 100 μM Cd^{2+}, respectively. At the high concentration of 1 mM, Cu^{2+} completely inhibited cross-linking. Next, to examine the mode of inhibition, a steady-state kinetic study was undertaken under the conditions in which the concentration of ligand [^{125}I]ET-1 was varied from 1 to 15 pM (Fig. 2). As expected from the nonsaturated condition of binding reaction, the ET-1 binding increased as the concentration of [^{125}I]ET-1 increased (Fig. 2, control). Upon the addition of Cd^{2+} (10 μM), the same IC_{50}s were found for all the [^{125}I]ET-1 concentrations tested. These results suggested that Cd^{2+} inhibits the formation of ET-1-receptor complex in a noncompetitive manner with respect to ET-1. Similar results were obtained when [^{125}I]ET-2 and [^{125}I]ET-3 were used as ligands (data not shown).

The effect of a chelating agent on the Cd^{2+} inhibition was examined, particularly to see if the reagent can prevent the inhibition and reactivate the Cd^{2+}-inactivated ET receptors. The experiments were carried out for membrane-associated receptor and solubilized receptor. With 0.1 mM Cd^{2+}, the binding of [^{125}I]ET-1 to either of the receptors were inhibited by more than 90% (Fig. 3). However, when 10 mM EDTA was incubated with either of the receptors, no difference in inhibition

Table I

Effect of divalent metal ions on ET-1 binding

Metals (10 mM)	Conc. (mM)	ET-1 binding (%)
none		100
Fe	10	100
Ca	10	91
Zn	10	85
Mg	10	77
Ni	10	73
Co	10	70
Mn	10	69
Hg	10	30
Cu	1	11
	0.1	49
Cd	0.1	7
	0.01	56

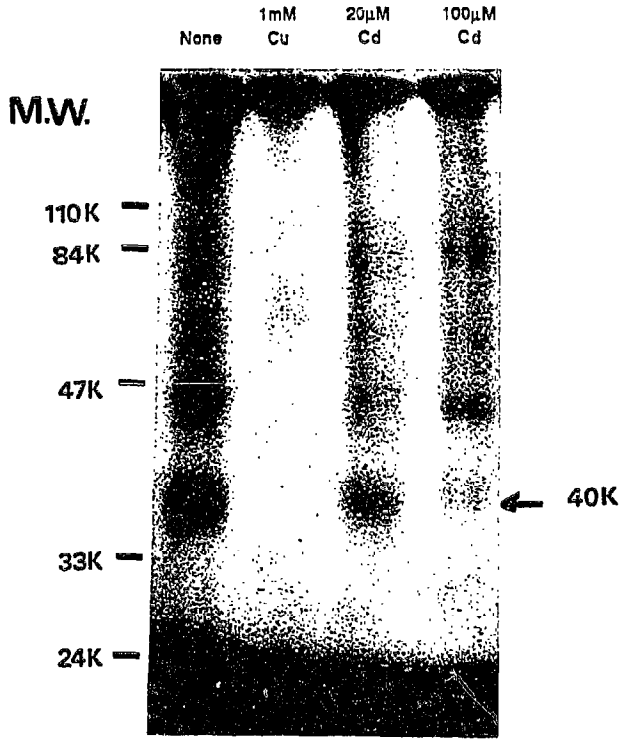

Fig. 1. Affinity labeling of ET receptor with [^{125}I]ET-1 in the presence or absence of Cd^{2+} or Cu^{2+}. Solubilized ET receptors (20 μg protein) were labeled by cross-linking with [^{125}I]ET-1 in the absence (lane 1) or presence of the indicated concentrations of $CuCl_2$ (lane 2) or $CdCl_2$ (lane 3,4). DSS (0.5 mM) was used as a cross-linker as reported previously [14]. Samples were subjected to SDS-PAGE under non-reducing conditions.

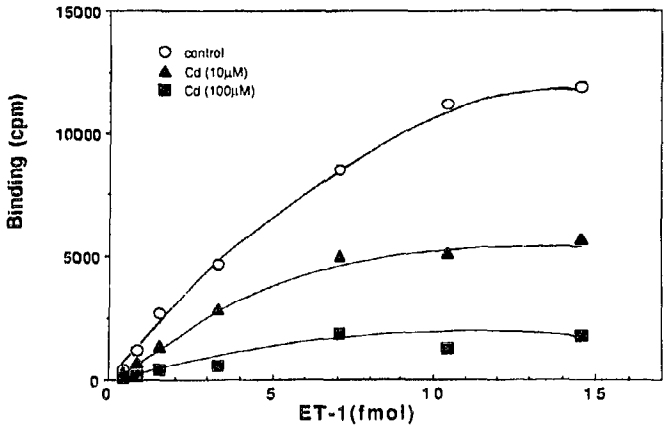

Fig. 2. Binding of solubilized ET receptor to [^{125}I]ET-1 in the presence or absence of $CdCl_2$. Solubilized ET receptor (2 μg) was incubated in the mixture as described in the Materials and Methods: no $CdCl_2$ (○—○), 10 μM $CdCl_2$ (▲—▲) or 100 μM $CdCl_2$ (■—■). ET receptors were assayed at different concentrations of [^{125}I]ET-1 as described in the Materials and Methods.

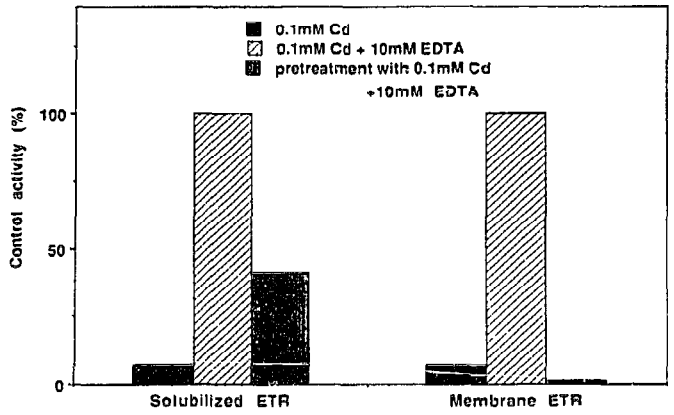

Fig. 3. Effect of EDTA on the Cd^{2+} inhibition of solubilized and membrane-bound ET receptors. Cd + EDTA represents a condition in which ET receptors were preincubated with 0.1 mM $CdCl_2$ and 10 mM EDTA. Pretreatment with Cd + EDTA represents experiments in which preincubation of ET receptor was performed with 0.1 mM $CdCl_2$, followed by the addition of 10 mM EDTA. Results are expressed as percentage of the binding activities of the control ET receptors which were incubated in the absence of $CdCl_2$ and EDTA.

was seen, indicating that EDTA could remove Cd^{2+} ion from the reaction mixture and the remaining large excess of free EDTA or Cd^{2+}/EDTA complex did not affect the binding activity. These results also suggest that the free Cd^{2+} ions bind to receptor molecule and prevent it to bind [^{125}I]ET-1. Furthermore, in order to understand the effect of EDTA on the Cd^{2+}-inactivated receptor, the following experiments were carried out. Both the membrane-associated receptor and the solubilized receptor were first incubated with 100 μM $CdCl_2$ for 15 min. 10 mM EDTA was then added to the mixture and the binding activity of receptors was measured to see if EDTA can remove the bound Cd^{2+} from receptor and restore the binding activity. As shown in Fig. 3, EDTA could recover 40–50% of binding activity for the solubilized receptors but it could not recover the activity for the membrane-associated receptors. The results indicate that the addition of EDTA after preincubation with Cd^{2+} was totally ineffective in reversing the Cd^{2+}-induced inhibition of membrane-bound receptors, but was partially effective in reversing the inhibition of the solubilized membranes.

There are several reports that described a hypotensive effect of Cd^{2+} on experimental animals. To relate the present findings of Cd^{2+}-induced inhibition of endothelin receptor to pharmacological hypotension, we examined the inhibitory effect of Cd^{2+} on rat aorta contraction by endothelin. As reported previously [1], 1 nM ET-1 induced an increase in the tension of the helical strip of aorta, which remained in a state of tension for at least 60 min (Fig. 4a). When Cd^{2+} (100 μM) was added, a gradual decrease in the tension occurred and, in about 15 min, the tension returned to the basal levels. Here, Cd^{2+} apparently reversed the effect of endothelin while Cd^{2+} by itself does not act as vasodilator. Next, when the aorta strip was preincubated in the medium containing 100 μM $CdCl_2$ for 10 min and when washed and soaked in the new Ringer solution containing 1 nM ET-1, the tension increased about 30% of the level of the untreated aorta strip. Curiously, the tension did not last long and it returned to basal levels within the second 60-min incubation period (Fig. 4b).

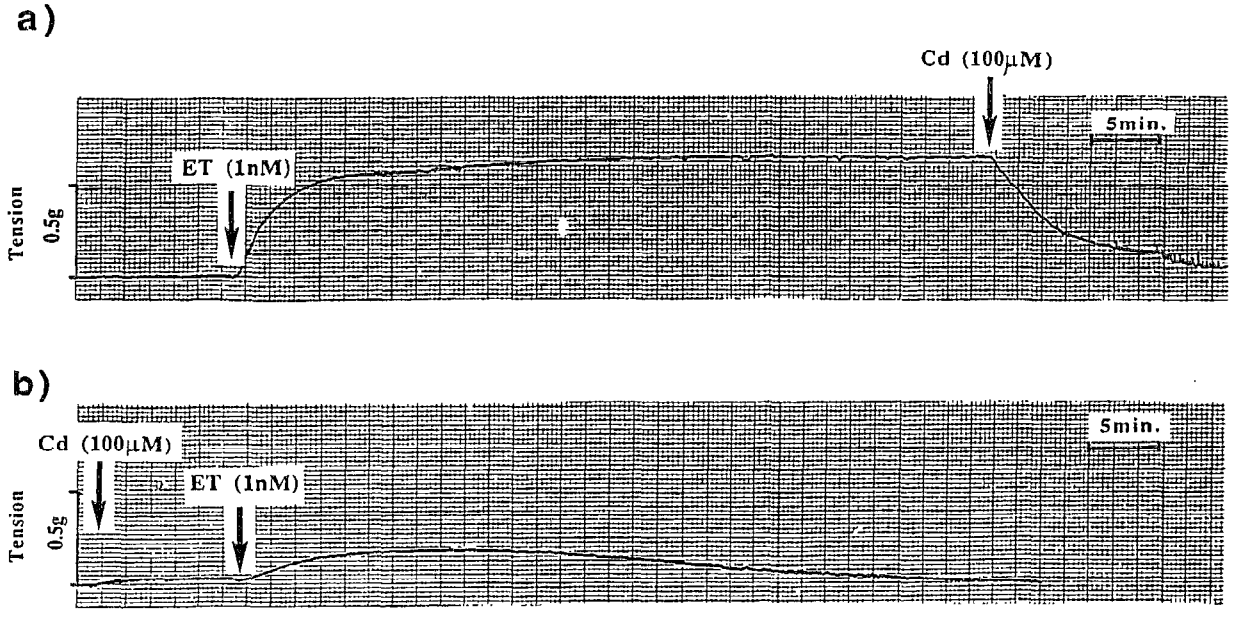

Fig. 4. Effect of $CdCl_2$ on contractile responses of rat aortic strips to ET-1. (a) Typical example of ET-1 induced contraction of rat aortic strips and the effect of $CdCl_2$. (b) A response curve to 1 nM ET-1 after treatment with 100 μM $CdCl_2$.

4. DISCUSSION

It has previously been demonstrated that Cd^{2+} inhibits the contractions of vascular muscles induced by noradrenaline, angiotensin and high-K^+ [15–18]. Recently, a few reports have indicated that Cd^{2+} also prevents the contractile action of ET-1 [19–21]. Inhibition of the inward fluxes of Ca^{2+} appeared to be the major mechanism responsible for the effect of Cd^{2+}. Therefore, they concluded from these studies that the attenuation of the ET-induced contraction by Cd^{2+} is due to a consequence of an inhibitory effect of Cd^{2+} on Ca^{2+}-channel and/or Na^+/Ca^{2+} exchange mechanisms. However, the present study has clearly demonstrated that Cd^{2+} interacts with the ET receptor and prevents binding to endothelin. Furthermore, we find that a 50% inhibition concentration of Cd^{2+} on ET-binding is in agreement with $IC_{50} = 15\ \mu M$ previously reported for by attenuation of the maximum response to ET-1 [19]. These results suggest that the attenuation of ET-induced contraction by Cd^{2+} may be a direct action via inhibition of ET-receptor. Irreversible inhibition of the membrane-associated receptor may be due to an allosteric conformational distortion of receptor molecules resulting from Cd^{2+} binding to membranes [18].

ET, like other vasoconstricting hormones such as phenylepinephrine, angiotensin II and histamine, acts on myocytes [1,2], but ET appears to mobilize Ca^{2+} in atrial cells better than the above compounds do. ET has been proposed to induce vasoconstriction by acting as an endogenous modulator of a dihydropyridine-sensitive, voltage-dependent Ca^{2+}-channel [4]. On the other hand, Vigne et al. [22] reported that the increase in Ca^{2+} concentration induced by ET was due to the mobilization of intracellular stores and due to the entry of Ca^{2+} into their sarcolemma via a pathway that does not utilize voltage-dependent L-Ca^{2+} channels. Indeed, ET stimulates the conversion of phosphatidyl inositol into inositol trisphosphate and diacylglycerol which mobilize Ca^{2+} from an intracellular pool, and activates protein kinase C, respectively [6,7,23,24].

The major role of ET is believed to be involved in the regulation of blood pressure and blood flow. Biological data suggest that ET and its receptor system may be involved in vasospasm and peripheral vasoconstriction that result in hypertension, renal failure and/or heart failure.

Our data demonstrate that the Cd^{2+} ion directly interacts with the ET receptor and inhibits the binding of endothelin. It may, however, be too early to relate this effect of Cd^{2+} on the inhibition of contraction, since Cd^{2+} apparently inhibits contraction caused by other vasoconstrictors. Cd^{2+} may inhibit various steps of signal transduction, the mechanism of which remains to be studied.

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